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Acidic Conditions Are Not Obligatory for Onset of Butanol Formation by *Clostridium beijerinckii* (Synonym, *C. butylicum*)

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Factors that may initiate the metabolic transition for butanol production were investigated in batch cultures of *Clostridium beijerinckii* (synonym, *Clostridium butylicum*) VPI 13436. Cultures maintained at pH 6.8 produced nearly as much butanol as those incubated without pH control, indicating that neither a change in the culture pH nor acid conditions per se are always required to initiate solvent formation. Acetate and butyrate levels at the onset of butanol production were dependent on the pH at which the cultures were maintained. Cultures maintained at pH 6.8 could be accelerated into solvent production by artificially lowering the pH to 5.0 or by the addition of acetate plus butyrate without a pH change (but neither acid alone was effective). Solvent production was associated with slower rates of growth and general metabolism, and it did not show a requirement for mature spore formation. We speculate that a slowdown in metabolism, which may be brought about by several conditions, is mechanistically related to the onset of butanol production. Extracts of solvent-producing cells contained acetoacetate decarboxylase activity as well as higher $NADP^+$-linked butanol dehydrogenase and lower hydrogenase activities than extracts of acid-producing cells. Solvent production did not appear to involve an enhanced ability to catalyze H_2 oxidation.

The acetone-isopropanol-butanol (or "solvent") fermentations by "*Clostridium butylicum*" and *Clostridium acetobutylicum* are characterized by a metabolic transition during which the acidogenic growth phase is succeeded by a solvent-producing phase whereby carbohydrate substrates and preformed acids are metabolized into neutral products with a consequent rise in the culture pH (18). The factors triggering the metabolic transition and the physiological states associated with the transition are poorly characterized; however, it is generally thought that solvent production represents a detoxifying response of the cell to an acidic pH which always precedes the onset of solvent production. Nearly all of the available evidence concerning the initiation of solvent formation is based on studies of *C. acetobutylicum* (1, 3, 10–12, 14). Solvent production in both batch and continuous cultures of *C. acetobutylicum* occurs at or below pH 5.2 (3, 11). The onset and extent of solvent production may be related to the levels of acetate or butyrate or both in the culture medium (3, 11). Since the distribution of weak acids such as acetate across the cytoplasmic membrane of bacterial cells is related to the ΔpH component of the proton motive force (16), the triggering mechanism may be linked to product excretion and membrane function. However, changes in culture densities, growth rate, and H_2 metabolism, as well as the onset of sporulation, also accompany the transition from acid to solvent production (14, 17). The causal relationships, if any, between these changes and the onset of solvent formation are unknown.

An understanding of the physiological factors governing the metabolic transition from acid to solvent production is likely to have practical applications in future process development and strain improvement. For example, strains of *Clostridium* lose the ability to produce solvents after repeated subculture (9, 18). This degenerative process could involve regulatory aberrations that result in the failure of the cell to respond to factor(s) triggering the onset of the metabolic transition. This type of cellular defect may be overcome if the triggering mechanism is known. In the present study, we examined the influence of culture pH and metabolic products on the onset of solvent production in batch cultures of *Clostridium beijerinckii* VPI 13436 (synonym, *C. butylicum* NRRL B592 [9]). We also studied changes in certain enzyme activities in extracts of acid- and solvent-producing cells.

MATERIALS AND METHODS

Organism and growth conditions. *C. beijerinckii* VPI 13436 (*C. butylicum* NRRL B592) was obtained from the Northern Regional Research Center, U.S. Department of Agriculture, Peoria, Ill. Batch cultures were grown in TYS medium containing (in grams [unless stated otherwise] per liter of deionized water): Na_2SO_4, 0.18; K_2HPO_4, 3.48; biotin, 0.01; *p*-aminobenzoic acid, 0.01; sucrose, 60; tryptone (Difco Laboratories, Detroit, Mich.), 1.0; yeast extract (Difco), 5.0; antifoam C (Sigma Chemical Co., St. Louis, Mo.), 0.5 ml; and 1.0 ml of a mineral stock solution (9). The medium was adjusted to pH 6.8 with HCl, autoclaved, and cooled under sterile nitrogen. Stock cultures were maintained as spore suspensions prepared from a 96-h batch culture in TYS medium which had been incubated at 30°C and stirred with a magnetic stirrer; sterile dimethyl sulfoxide was added to a final concentration of 5% (vol/vol), and 1.0-ml portions were stored in screw-capped, plastic vials in liquid nitrogen.

For each growth experiment, one vial of the spore suspension was thawed, immersed in a boiling water bath for 1 min, transferred immediately to 8 ml of chopped meat carbohydrate medium (13), and incubated statically at 35°C for 24 h. A 2-ml amount of this culture was transferred to 40 ml of TYS medium which was then incubated statically at 35°C for 16 h. This culture was used to inoculate 800 ml of TYS medium which was then incubated at 30°C and stirred with a magnetic stirrer. pH control was achieved by the automatic addition of 8 N KOH with a pH controller (New Brunswick Scientific Co., New Brunswick, N.J.). HCl (4 N) was used in experiments in which the culture pH was lowered artificially. Where indicated, potassium acetate or potassium butyrate was added from stock solutions (2.0 and 1.54 M, respectively) which had been cooled under nitrogen after autoclaving. Where indicated, 800-ml cultures were sparged with H_2 or N_2 at a flow rate of 35 ml/min.

The onset of solvent formation was defined as the time at which the butanol concentration reached 1 mM.

Analytical methods. Growth was monitored at 550 nm with a Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, N.Y.) with 1.2-cm cuvettes. Cultures were diluted when necessary so that the absorbance at 550 nm (A_{550}) did not exceed 0.3. Acid and solvent products were quantitated by gas chromatography of culture supernatants (9, 13). Sucrose was determined by the anthrone method (2). Protein was estimated by the Bio-Rad dye binding assay (Bio-Rad Laboratories, Richmond, Calif.) with bovine gamma globulin as a standard. Polyacrylamide gel electrophoresis was performed as described by Davis (7),but with the sample layered on top of the polymerized gel. One milligram of crude extract protein was loaded per gel tube.

Enzyme assay. Hydrogenase activity was measured spectrophotometrically at 604 nm in anaerobic cuvettes containing an H_2 atmosphere and a reaction mixture of 50 mM Tris-hydrochloride (pH 8.0), 10 mM methyl viologen (ϵ_{604} = 13.6 mM^{-1} cm^{-1} [15]), and 2 mg of bovine serum albumin per ml in a final volume of 0.6 ml. Acetoacetate decarboxylase activity was measured as described by Davies (6). Butanol dehydrogenase was assayed as described by Bergmeyer et al. (4), substituting 0.02 ml of *n*-butanol for ethanol in a reaction mixture of 0.6 ml. One unit of activity was defined as 1 μmol of substrate utilized or product formed per min at 20°C. The activity stain for butanol dehydrogenase contained 0.1 M glycine-NaOH (pH 9.0), 1 mM NAD^+ or $NADP^+$, 1 mM iodonitrotetrazolium violet, 0.1 mM phenazine methosulfate, and 3.7% (vol/vol) *n*-butanol. Gels were incubated in the dark at 20°C for 1 h.

Extracts from acid- and solvent-producing cells were prepared from 7-liter TYS batch cultures harvested by centrifugation at 10,000 × *g* for 30 min at 4°C when the A_{550} reached 0.96 and 2.2, respectively. Cell pellets were washed once with 50 mM Tris-hydrochloride (pH 8.0), equilibrated with nitrogen, and stored in liquid nitrogen. Cell pellets were thawed in 50 mM Tris-hydrochloride (pH 8.0) buffer containing 1 mg of lysozyme (Sigma) per ml, using 3 ml of buffer per g (wet weight), and equilibrated with H_2 by 10 cycles of evacuation and filling. Cell suspensions were stirred at 20°C for 90 min and then centrifuged at 37,000 × *g* for 30 min at 4°C. The supernatant (crude extract) was decanted and stored in liquid nitrogen.

RESULTS

Solvent formation in batch cultures without pH control and in those maintained at pH 6.8. Solvent production in batch cultures of *C. beijerinckii* VPI 13436 began when the culture pH reached 4.5 to 4.6 (Fig. 1). Batch cultures dis-

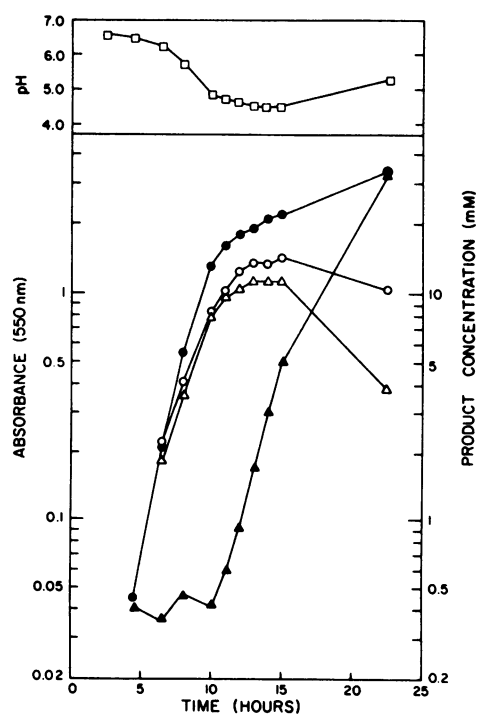

FIG. 1. Solvent production by *C. beijerinckii* VPI 13436 in TYS medium without pH control. Symbols: ●, A_{550}; ○, acetate; △, butyrate; ▲, butanol; □, pH.

played the "pH break" phenomenon characteristic of the solvent fermentation and produced 100 to 120 mM butanol after 120 h of incubation. However, solvent production (up to 90 mM butanol after 120 h of incubation) also occurred in batch cultures maintained at pH 6.8 (Fig. 2). Thus, neither a change in the culture pH nor acid conditions per se was required to initiate solvent production.

Several differences in the fermentation patterns were observed under these two culture conditions. In batch cultures maintained at pH 6.8, the cells showed normal motility until the A_{550} reached ca. 2.9. Butanol formation began ca. 1.5 h later as maximum growth was achieved (A_{550} = ca. 3.2). At this time the culture contained a mixed population of motile and sluggishly motile cells, and the acetate and butyrate concentrations reached ca. 37 and 35 mM, respectively. The cell population was virtually nonmotile when the butanol concentration reached ca. 4 mM. Solvent-producing cells at pH 6.8 were generally longer than those observed during the logarithmic phase of growth. Cultures maintained at pH 6.8 did not show signs of mature spore formation. Acetate, but not butyrate, continued to accumulate during solvent production, reaching a final concentration of over 100 mM.

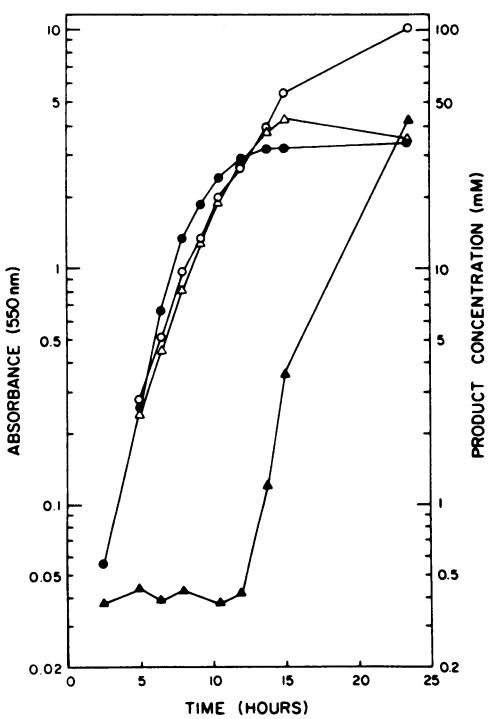

FIG. 2. Solvent production by *C. beijerinckii* VPI 13436 in TYS medium maintained at pH 6.8. Symbols: ●, A_{550}, ○, acetate; △, butyrate; ▲, butanol.

In batch cultures without pH control, butanol formation began at lower cell densities (A_{550} = ca. 1.8) and acid levels (ca. 12 mM acetate and 10 mM butyrate). The acidogenic growth phase was characterized by highly motile, vegetative cells which became sluggishly motile when the pH had fallen to ca. 4.7. During the next 3 h of incubation, the cultures produced both acids (the culture pH decreased further to the minimum value of 4.5) and solvents (the butanol concentration reached 5 mM). The cells present at pH 4.5 were virtually nonmotile; however, motility resumed immediately if the culture was adjusted to pH 6.3. In the absence of an artificial pH shift, good cell motility resumed after ca. 15 mM butanol was produced and the culture pH had risen to ca. 4.8. With further incubation, the cells assumed the swollen, "clostridial" form associated with the early stages of sporulation. Refractile spores were observed after ca. 28 h of incubation.

Effect of metabolic products on solvent production. Acetate or butyrate or both were added to pH 6.8 cultures of various cell densities to give concentrations at least as high as those present at the onset of solvent production in unsupplemented control fermenters (Table 1). Acetate or butyrate added separately in concentrations approximating those present at the onset of butanol production in control fermenters did not shorten the lag time (i.e., the incubation time required for butanol formation to begin from the cell densities present at the time of acid addition) as compared to the control ferementers. Acetate plus butyrate added together at A_{550} = 2.0 or 1.0 shortened the lag time by 1.4 h in each case. The effect did not appear to result from increased ionic strength or potassium concentration, since the addition of an equivalent concentration of KCl had no effect (data not shown). Acetate or butyrate alone added in levels similar to the total concentration of acetate plus butyrate added in other experiments had little effect on lag time, suggesting that the effect of acetate plus butyrate was not a nonspecific response to any weak acid. Acid production continued after each addition was made, but the rates of acid production as well as growth were slower than those present before each addition (data not shown). The final concentrations of butanol in butyrate-supplemented fermenters (ca. 155 mM butanol after 120 h of incubation) were higher than in control fermenters (ca. 91 mM butanol).

Batch cultures without pH control sparged with H_2 or N_2 began to produce solvents at comparable cell densities (A_{550} = ca. 1.8) and pH values (4.6 to 4.7) and formed similar amounts of butanol after 48 h of incubation (114

TABLE 1. Effect of acetate and butyrate on butanol production in batch cultures of *C. beijerinckii* VPI 13436 maintained at pH 6.8

Addition	A_{550} when acids added	Acid concn (mM) added[a]		Acid concn (mM) at onset of butanol production		A_{550}[b]	Lag time[c]
		Acetate	Butyrate	Acetate	Butyrate		
None	NA[d]	NA	NA	37	35	3.2	NA
Acetate	2.3	37	NA	75	38	3.4	3.6 (3.2)
Butyrate	2.0	NA	29	44	59	3.2	3.7 (3.8)
Acetate plus butyrate	2.0	41	26	66	50	2.5	2.5 (3.8)
Butyrate	2.2	NA	63	26	82	2.5	3.1 (3.3)
Acetate	2.1	71	NA	96	31	2.5	4.3 (3.5)
Acetate plus butyrate	1.0	47	29	75	51	2.8	4.5 (5.9)

[a] Acetate and butyrate concentrations before additions were 15 and 14 mM, respectively, at A_{550} = 2.0 and 8 and 6 mM, respectively, at A_{550} = 1.0.

[b] At onset of butanol production.

[c] Hours between addition of acids and the onset of butanol production. Time in parentheses is the time required for butanol formation to occur, starting from the indicated cell densities in unsupplemented fermenters maintained continuously at pH 6.8.

[d] NA, Not applicable.

and 104 mM, respectively). H_2 at 101 kPa did not appear to influence the onset or extent of solvent production.

Effect of pH on solvent production. The relationship between cell density and ability to produce solvents was examined in batch cultures maintained at pH 6.8 and then shifted to pH 5.0 (Table 2, experiments 1 through 4). Solvent formation in cultures shifted when the A_{550} reached 0.5, 1.0, or 2.0 began at lower cell densities and shorter incubation times than that in control fermenters maintained at pH 6.8.

TABLE 2. Effect of pH shifts on solvent production by *C. beijerinckii* VPI 13436[a]

Expt	A_{550} at shift	Hours at pH 5.0	A_{550} at onset of butanol production	Lag time[b]	Butanol (mM) at 120 h
1	0.5	NA[c]	1.7	5.9 (9.3)	121
2	1.0	NA	1.6	3.8 (8.0)	118
3	2.0	NA	2.2	2.6 (5.0)	139
4	3.1	NA	NA	NA (0)	0.5
5	1.0	0.25	2.5	5.7 (8.0)	132
6	1.0	0.50	2.0	12.7 (8.0)	103
7	1.0	1.0	1.9	10.9 (8.0)	137

[a] Cultures were maintained at pH 6.8 until they reached the indicated cell densities. Experiments 1 through 4 were lowered to pH 5.0 and incubated without further pH control. Experiments 5 through 7 were lowered to pH 5.0, incubated for the indicated times, and then returned to and maintained at pH 6.8.

[b] Hours after beginning of pH shift until the onset of butanol production. Time in parentheses is the time required for butanol production to occur, starting from the indicated cell densities in control fermenters maintained continuously at pH 6.8.

[c] NA, Not applicable.

Cultures shifted to pH 5.0 at an A_{550} between 0.5 and 2.0 produced 118 to 139 mM butanol, whereas those shifted at an A_{550} of 3.1 did not form appreciable levels of solvents.

Since pH 5.0 treatment appeared to have a favorable influence on the onset of solvent production when the culture density (A_{550}) was 2.0 or below, we wished to determine whether continuous exposure to acid conditions was required to elicit this effect (Table 2, experiments 5 through 7). Cultures at A_{550} = 1.0 which were treated for 0.25, 0.50, or 1.0 h at pH 5.0 required longer incubation times before solvent formation began than the corresponding fermenter (experiment 2) which was not returned to pH 6.8 after pH 5.0 treatment. Cultures treated for 0.5 and 1.0 h at pH 5.0 had lag times longer than even control fermenters maintained continuously at pH 6.8. However, butanol production began at somewhat lower culture densities as compared to control fermenters.

Since the total acid concentrations and culture densities present at the onset of butanol formation were much higher in cultures maintained at pH 6.8 (Fig. 1 and 2), we examined product formation in batch cultures maintained at other pH values (Fig. 3). The levels of total, dissociated, and undissociated acetate were 3.4-, 4.7-, and 8.8-fold higher at pH 6.8 than at pH 5.2, respectively. The levels of total, dissociated, and undissociated butyrate were 3.6-, 5.0-, and 8.1-fold higher at pH 6.8 than at pH 5.2, respectively. (The degree of dissociation was calculated from the pK_a's of the acids.) The culture densities (A_{550}) present at the onset of butanol production were 1.6, 2.5, and 3.2 at pH values of 5.2, 6.0, and 6.8, respectively.

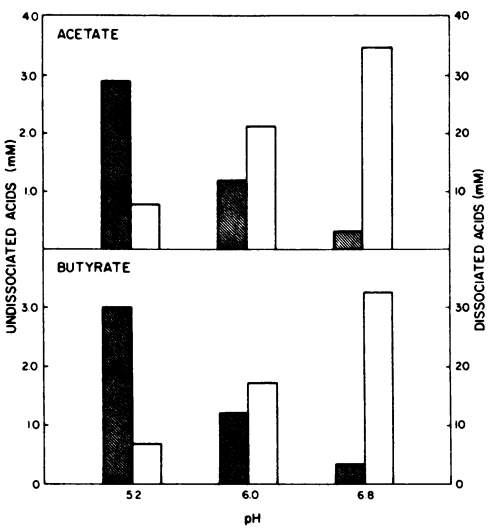

FIG. 3. Undissociated (closed figures) and dissociated (open figures) acid levels at the onset of butanol production in batch cultures of *C. beijerinckii* VPI 13436 maintained at various pH values.

Metabolic rates during acid and solvent production. The rates of sucrose catabolism in uncontrolled and constant pH batch cultures were measured before and after the onset of solvent production (Table 3). Growth at pH values near neutrality resulted in higher catabolic rates. In each case, catabolic rates were at least 40% lower during solvent rather than acid production.

Enzyme activities in acid- and solvent-producing cells. The activities of hydrogenase, butanol dehydrogenase, and acetoacetate decarboxylase were measured in extracts of cells harvested before and after the pH break (Table 4). Hydrogen uptake and evolution activities were lower in solvent-producing cells, whereas the ratio of uptake to evolution was constant. Butanol dehydrogenase activity was increased in solvent-producing cells and appeared to require $NADP^+$ as a coenzyme. This activity could be measured in the physiological direction of NAD(P)H oxidation, but assay conditions showing proportionality of initial reaction rate to enzyme concentration were not established. Also, extracts showed high endogenous rates of NADH oxidation. In situ activity stains for butanol dehydrogenase on polyacrylamide gels after electrophoresis of crude extracts supported the idea that butanol dehydrogenase activity was $NADP^+$ linked. Moreover, multiple forms of the enzyme appear to be present (R_f values of 0.59, 0.81, and 0.85). Acetoacetate decarboxylase activity was detected only in extracts of solvent-producing cells. Attempts to demonstrate coenzyme A-dependent butyraldehyde dehydrogenase activity with NAD^+ or $NADP^+$ were not successful.

TABLE 3. Rates of sucrose catabolism in batch cultures of *C. beijerinckii* VPI 13436

Culture condition	Catabolic rates[a] during:	
	Acid production	Butanol production
pH uncontrolled	1.55	0.93
Constant pH 5.2	1.67	0.76
Constant pH 6.0	2.50	1.01
Constant pH 6.8	3.16	1.35

[a] Micromoles of sucrose catabolized per hour per milliliter per A_{550} unit.

TABLE 4. Enzyme activities in acid- and solvent-producing cells of *C. beijerinckii* VPI 13436

Enzyme	Substrate	U/mg[a] in cells producing:	
		Acids	Solvents
Hydrogenase	Reduced methyl viologen-H^+	13.6	6.1
	H_2-methyl viologen	27.2	11.8
Butanol dehydrogenase	Butanol-$NADP^+$	0.00099	0.012
	Butanol-NAD^+	—[b]	—
Acetoacetate decarboxylase	Acetoacetate	—	0.83

[a] Micromoles of substrate utilized or product formed per minute per milligram of protein.
[b] —, No activity detected.

DISCUSSION

Solvent production has not been reported to occur in cultures maintained at a constant pH near neutrality. Solvent production in chemostats (1, 3) or the onset of solvent production in batch cultures (11, 18) of *C. acetobutylicum* occurs at or below pH 5.2. An examination of the solvent fermentation under two strikingly different culture conditions, namely, at a constant pH of 6.8 and in cultures without pH control, provides a means to assess the regulatory processes integral to the transition from acid to solvent production.

No specific values of acid concentration (dissociated, undissociated, or total levels), culture density, or pH could be correlated with the onset of solvent formation. Based on pH shift and acid addition experiments, several related parameters (acidic pH, high weak acid concentrations, and high cell densities) may be involved, perhaps in specific combinations, in the mechanism for triggering solvent formation by *C. beijerinckii*. However, these treatments can only shorten the incubation time, which is time elapsed before solvent formation begins, by about 1 h. Further experiments which monitor

the appearance of enzyme activities related to solvent production may better define the physiological changes in response to specific treatments. Both pH shift and acid additions increased the levels of undissociated weak acids present in the culture medium. If weak acids such as acetate and butyrate cross the cytoplasmic membrane of *C. beijerinckii* in their undissociated forms, as is thought to occur in other organisms (16, 19), it seems likely that the accumulation of weak acids both inside and outside the cell during growth would influence the regulation of the internal pH and the magnitude of the transmembrane proton motive force. The slowdown of general metabolism and motility at the onset of solvent formation could be interpreted in terms of weakened membrane functions resulting from a diminished proton motive force (20). Changes in the intracellular pH may also influence the oxidation-reduction potential and those metabolic functions regulated by intracellular oxidation-reduction potential. However, our acid addition experiments in which acetate and butyrate together (rather than acetate or butyrate alone at an equivalent total acid concentration) influenced the onset of solvent production suggest that the effect of weak acid products may be specifically involved in the metabolic pathways for product formation. For example, acetate addition may partially inhibit acetate kinase activity and result in elevated intracellular concentrations of acetyl phosphate, which may inhibit pyruvate oxidation (5). Such a feedback mechanism may participate in the slowdown of general metabolism observed during solvent production.

Solvent production in batch cultures of *C. acetobutylicum* is temporally associated with the early stages of sporulation (14), suggesting that the initiation of both sporulation and solvent production may involve a shared regulatory mechanism. In the present study, the slowdown of general metabolism during solvent production may reflect a similar situation, since sporulation is often initiated after nutrient deprivation, with concomitant slower rates of growth (8). However, even the early, "clostridial" stage of sporulation was not obligatorily linked to solvent production in batch cultures maintained at pH 6.8.

Rerouting of both carbon and electron flow must accompany the transition from acid to solvent production. This substrate channeling in part involves elevated activities of butanol dehydrogenase and acetoacetate decarboxylase. There do not appear to be qualitative changes in hydrogenase to indicate an enhanced capacity for H_2 oxidation during solvent formation. Rates of H_2 evolution from whole cells of *C. beijerinckii* are lowest during solvent formation; such cells do not show net H_2 uptake when assayed manometrically (data not shown). The lower rates of H_2 production could involve several factors: (i) lower rates of general metabolism that diminish the supply of electrons, (ii) decreased levels of hydrogenase, and (iii) enhanced electron flow from reduced ferredoxin to $NAD(P)^+$ rather than to hydrogenase. Reduction of $NAD(P)^+$ by ferredoxin may occur through the action of ferredoxin:$NAD(P)^+$ oxidoreductase (17); such an enzyme may provide NADPH for butanol formation.

The yield of solvents by industrial strains of *C. acetobutylicum* is about 30% (21), which is calculated as the weight of solvents produced versus the weight of sugars metabolized (it is equivalent to a yield of about 80% on a molar basis, i.e., based on moles of solvents produced versus moles of hexose equivalents metabolized). The yield of butanol is about 18% on a weight basis and 43% on a molar basis. In this study, no effort was made to optimize the yield because we focused on parameters which might effect the transition of cells from acid production to solvent production. However, the yield of butanol by *C. beijerinckii* was at least 15% on a weight basis or 34% on a molar basis under our normal culture conditions.

ACKNOWLEDGMENTS

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